HINDUSTAN ANTIBIOTICS

Bulletin

VOL. 1 MAY 1959 No. 4

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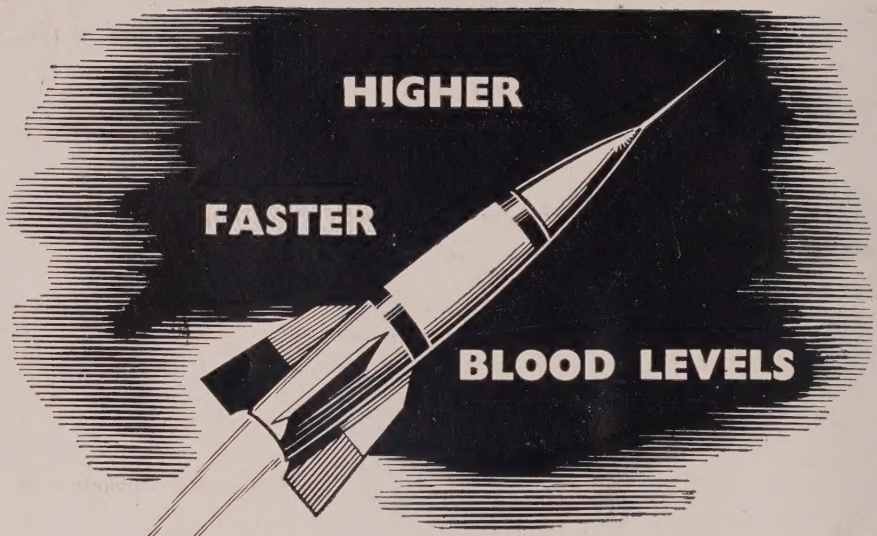

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HINDUSTAN ANTIBIOTICS
Bulletin

Vol. 1 **May 1959** **No. 4**

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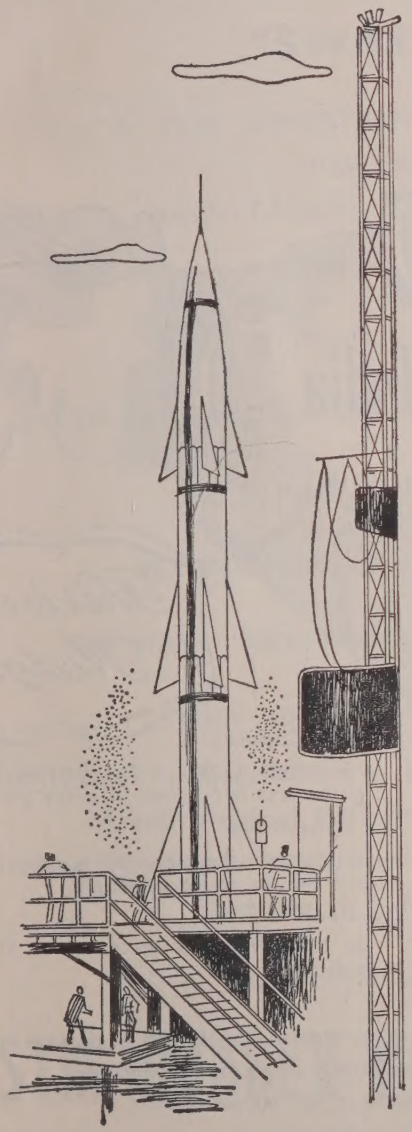

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K. 47 (a)

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Mycotic Infections and Antibiotics

FUNGI inciting superficial and deep mycoses in man are well known. The sufferings they cause are considerable, and until recently several of the diseases were considered incurable. Fortunately some of the dreaded ones like the South American blastomycosis, North American blastomycosis, sporotrichosis, histoplasmosis, etc., are endemic, restricted to certain parts of the world and have a slow rate of spread. However, with the introduction of potent antibacterial antibiotics, the diseases incited by fungi are on the increase requiring serious attention. The removal of the competing bacterial counterpart by antibiotics in a natural environment, gives place to uninhibited growth of fungi like *Candida albicans* (Robin) Berkhout. Moniliasis or candidiasis which was encountered only in rare cases has now become fairly common and is posing a big problem in the use of broad spectrum antibacterial antibiotics. There have been several cases of fatal infections caused by *Candida albicans* following tetracycline therapy. The general yeast flora of the gastrointestinal tract has been shown to increase accompanying administration of tetracyclines. The need for antifungal substances is, therefore, all the more evident.

Compared with the success of antibiotic treatment of many bacterial diseases of man, the results in the case of fungal infections have not been spectacular. This is due primarily to the nature of the disease inciting organisms which are slow growing, produce resting bodies, chlamydospores, sphaerules, etc., developing in tissues not easily amenable to antibiotic treatment. Moreover, most of the antifungal antibiotics are highly toxic and there are very few of real therapeutic value.

Among the antifungal antibiotics, most of the important ones are of the polyenic type produced by species of *Streptomyces*. Nystatin, rimocidin, antimycoin, chromin, eurocidin, fungichromin, fungichromatin, filipin, flavacid, mediocidin, candicidin, amphotericin A and B, candidin, trichomycin, candimycin are some of the important polyenic antibiotics. In the screening programme for antibiotics, investigators use many of the human fungal pathogens and, of the several antibiotics with antifungal spectra only nystatin, amphotericin B, trichomycin and griseofulvin have been used therapeutically.

The superficial mycoses incited by species of *Trichophyton*, *Microsporum* and *Epidermophyton* are well known in the tropics. Chemicals like iodine, undecylenic acid, etc., have been used with some success in controlling them. Antibiotics provided for the first time new methods of treatment as external applications in various formulations. Candicidin, ascocin and other antibiotics have been used in the control of tænia capitis and tænia pedis. The recent finding of the therapeutic use of griseofulvin, an antibiotic known for a long time for causing peculiar malformations in other fungi, deserves mention. In low concentrations, this antibiotic (called "curling factor") produces curling of mycelia of fungi. When administered internally it is claimed to suppress superficial mycoses.

In the control of deep mycoses antibiotics have been of real value. Some of the fungi causing histoplasmosis, blastomycosis, sporotrichosis, etc., have peculiar growth forms. *In vitro*, they have a mycelial form with characteristic spore types, while *in vivo* they get transformed into an yeast phase

within the host cells. Earlier investigators confused these yeast cells for protozoans. The treatment for these deadly diseases has until recently been the use of chemicals such as iodides of heavy metals, neoarsephenamine, stilbamine glucoside, stilbamidine, etc., which are on the borderline of killing the host also. The antibiotic amphotericin B provided for the first time a powerful tool for the control of these systemic mycoses. Several cases of histoplasmosis have been successfully treated without having any after-effects on the patient. In animal tests oral doses of amphotericin B protected mice against *Coccidioides immitis*, and *Sporotrichum schenkii*. Antibiotics are, therefore, holding out a promise in combating mycotic infections of man which, at one time, were considered incurable. There is an increasing need for antifungal antibiotics of real therapeutic value and they are bound to change the future outlook on the treatment of human mycoses.

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(Sd.) S. T. Raja,
for HINDUSTAN ANTIBIOTICS LTD.,
Pimpri, Poona Dist.

Antibiotics and Plant Disease Control

S. K. MENON, Ph.D. (Ohio)

LITERALLY hundreds of compounds are available today which aid in crop protection as fumigants, sprays, dusts and systemics. The more important of these are sulphur, salts of copper and organic acids, organic mercurials, quinones and heterocyclic nitrogen compounds. In spite of the availability of such a wide variety of compounds, over 50 per cent of the plant diseases are not, however, satisfactorily controlled. The discovery of penicillin in 1929, its isolation and successful use in the control of human pathogens since the early 1940's, greatly stimulated research on the application of antibiotics for plant disease control. Attempts to use antibiotics for plant disease control, began a decade ago and Zaumeyer and co-workers (1952) of the United States Department of Agriculture were among the first to use them under field conditions. Excellent control of certain diseases, especially those incited by bacteria has been achieved from the large commercial scale use of antibiotics, mainly in the United States.

Although a number of antibiotics are being investigated, those derived from the genus *Streptomyces* have so far shown greater promise in plant disease control on field scale. Some of the better known compounds and formulations are streptomycin, Agrimycin, Agristrep, Phytomycin, Acco-Streptomycin and actidione (cycloheximide).

Control of Bacterial Diseases

Fire-blight of apples and pears caused by the bacterium *Eriwinia amylovora*, has been successfully controlled with three applications of streptomycin at 120 parts per million during blossoming period. The fire-blight bacterium acquires a strong measure of resistance when exposed to streptomycin for several generations in culture. This development of resistance could be suppressed by the addition of a small quantity of oxytetracycline. A combination of streptomycin and oxytetracycline in 90 : 10 ratio (Agrimycin) developed at the Pfizer Laboratories, effectively controls fire-blight at concentrations as low as 30 p.p.m. and forestalls development of resistance in the field.

The combination of streptomycin and oxytetracycline acts synergistically by some mechanism not yet fully understood. Investigations at Boyce Thompson Institute in New York revealed that Agrimycin also has synergistic action with certain insoluble copper compounds. A combination of dilute copper sulphate solution added to streptomycin at sub-lethal doses of both materials, is toxic to bacteria. This opens up an interesting field of study and considerably widens the scope of improving the efficacy of the conventional types of fungicides by combining them with antibiotics.

Seedpiece decay of cut potatoes incited by *Eriwinia atroseptica* and *Pseudomonas fluorescens* has been successfully controlled by dipping freshly cut potato seed-pieces in aqueous solution containing 250 p.p.m. streptomycin sulphate. With the suppression of the bacteria, it was observed that the seed pieces became very susceptible to the attack of fungi such as *Fusarium* and *Phoma* species.

Another bacterial disease satisfactorily controlled under field conditions with five applications of streptomycin at 400 p.p.m. is the bacterial spot of tomato and pepper incited by *Xanthomonas vesicatoria*. The yield in the treated plots was 50 per cent more than in the untreated plots.

Tobacco seedlings in seed beds sprayed with a solution containing 200 p.p.m. Agrimycin, protected the plants from the bacteria causing "Wild fire" (*Pseudomonas tabaci*).

Control of Diseases caused by Fungi

The field evaluation of antibacterial antibiotics has shown promising results but the antifungal antibiotics so far known are not as effective in controlling plant diseases. A vast majority of plant diseases is caused by fungi and of these a substantial number have not been controlled due to practical difficulties. The control of a large number of fungus diseases with antibiotics may not be accomplished as rapidly as that of the bacterial diseases. This is due to the diversity of the fungi causing diseases, their varied response to antibiotics and the different host-parasite relationships. Among the several antifungal antibiotics actidione (cycloheximide) and griseofulvin have shown some promise, having a wide *in vitro* antifungal spectrum.

Actidione, produced by *Streptomyces griseus*, has a broad anti-fungal spectrum and is active at low concentrations against most fungi. As early as 1948, it was shown that actidione spray at a concentration of 1 to 5 p.p.m. controlled powdery mildew of beans caused by *Erysiphe polygoni*. Actidione is also being used in fruit disease control. Cherry leaf spot caused by *Coccomyces hiemalis*, can be prevented by the application of the antibiotic at 1 to 2 p.p.m. at various periods during the growing season. A rapid seed treatment of wheat with 0.5 to 1 per cent of actidione is reported to give almost complete control of covered smut of wheat. In greenhouse tests, application of actidione to wheat plants have also been found to give some degree of control against rust, *Puccinia graminis* var. *tritici*. Extensive use of the antibiotic has probably been retarded by the narrow margin between the levels required for disease control and those causing phytotoxicity.

Griseofulvin, produced by *Penicillium nigricans*, has been known for some years as a systemic antifungal antibiotic of some promise in controlling *Fusarium* and other root-rot pathogens. Griseofulvin is fungistatic and induces the formation of morphological abnormalities of the mycelium in the cells containing it, thus preventing the fungus passing from cell to cell through the plant membranes.

Streptomycin formulations have also been found to possess antifungal properties though they have no activity against fungi *in vitro*. Sprays of Agrimycin, Agristrep and Phytomycin containing 100 p.p.m. of streptomycin gave excellent control of downy mildew of beans (*Phytophthora phaseoli*) and tobacco (*Perenospora tabacina*) The addition of neutral copper to the antibiotic considerably increased its efficacy. This mixture has also been found to give fairly good control of late blight of potatoes (*Phytophthora infestans*).

Leben, Arny and Keitt have reported from greenhouse as well as field tests that seed treatment with crude Helixin B (from *Streptomyces* sp.) could control blight of oats, caused by *Helminthosporium victoriae* and that of barley caused by *H. sativum*.

Control of Virus Diseases

Only limited investigations have so far been conducted on the inhibition of plant viruses by antibiotics. Recently it has been demonstrated that certain antibiotics are of value in the treatment of peach rosette virus. Infection by tobacco mosaic virus has been reported to be inhibited by using

Noformicin (produced by *Nocardia formica*) spray at a concentration of 125 p.p.m.

Transport of Antibiotics in Plants

Better transport of the antibiotics within plants means greater efficiency and the possibility of fewer applications. It also affords protection to new growths subsequent to spraying. The absorption of antibiotics and their subsequent translocation within plants vary with both plant species and the antibiotic. Antibiotics such as penicillin, streptomycin, oxytetracycline and chlortetracycline are known to be absorbed and translocated within plants. Zaumeyer and co-workers have shown that streptomycin applied in lanolin paste to the stems of bean plants, was readily absorbed and translocated to the primary leaves. As much as 67 per cent of the total streptomycin applied to the stem was translocated to the primary leaves as free streptomycin during the first 5 days immediately following treatment.

Several antibiotics are absorbed by seeds or roots and translocated throughout the plant where they may act as systemic protectants against pathogens. For instance, when the roots of oat plants are immersed for 7 days in a solution containing 10-50 μg/ml of griseofulvin the guttation fluid may contain 0.1 to 1 μg. of the antibiotic, which continues to appear in the guttation fluid 3-4 weeks after the plants have been removed from the antibiotic containing solution. Recently Crowdy and associates presented quantitative data on the uptake of griseofulvin through roots of broad bean (*Vicia* sp.) and tomato. They established that griseofulvin was taken up and translocated unchanged. They were successful in isolating the antibiotic quantitatively from different parts of the plant, and the amount taken up was found to be linearly related to the amount of water transpired.

Treating seeds with antibiotics before planting, renders the resulting seedlings free from certain fungal and bacterial infections. Cucumber seeds soaked in 1:10,000 to 1:1,000,000 solution of streptomycin absorb enough antibiotic to protect them from *Pseudomonas lachrymans*, the causal agent of angular leaf spot.

Almost all the antibiotics that have been investigated in relation to translocation in plants have been shown to move in an upward direction. Antibiotics which when sprayed on foliage would move downward and into the roots might open up possibilities for the control of root-rot pathogens. Recently Gray in his investigations at the Merck Research Laboratories found that streptothricin and pleocidin, sprayed on intermediate leaves of bean or tobacco plants at 1 per cent concentration, were readily translocated in good amounts, downward to older leaves and upward to the young leaves. Further, these two antibiotics were found to be translocated downward through the xylem. This is contrary to the available information on the downward movements of other organic solutes which move mainly in the phloem tissue.

In some instances, translocation of the antibiotic seems to be dependent on the method of application. When sprayed, streptomycin, dihydrostreptomycin, neomycin, bacitracin and actinomycin fail to move out of bean and tobacco leaves, in detectable amounts. However, some of these antibiotics were translocated when one leaf was immersed in a solution of any one of the antibiotics.

Temperature also influences translocation of antibiotics in plants. It has been reported that unless the atmospheric temperature is below 70°F, streptomycin would be almost ineffective against fireblight of apple and pear. Tests revealed that temperature of 70°F or above did not prevent streptomycin from entering the leaves but translocation was not effective.

The efficacy of the antibiotics could be substantially increased by incorporation of

compounds like indole-3-acetic acid and ethyl-3-acetate. This property is also shared by a large number of compounds which are considered plant growth regulators. The enhanced disease control efficiency is not due to the action of these compounds directly on the pathogen but rather due to their effect on the host metabolism (increased water uptake, increased permeability of the cell membranes, faster rate of growth and speedy maturation due to increased metabolic rate). The effectiveness of streptomycin against bacterial blight of beans (*Xanthomonas phaseoli*) could be increased by the addition of 1 per cent glycerine. It appears that longer the exposure of plant surfaces to streptomycin in a wet form, the better the protection against bacterial agents. Glycerine being a humectant serves this purpose efficiently.

Mode of Action of Antibiotics

At present our knowledge of the mechanism by which antibiotics bring about control of plant diseases is far from adequate. While it is generally assumed that antibiotics act directly on the pathogen, this explanation does not cover all the results so far claimed. Recent reports that streptomycin shows *in vivo* activity against pathogens (e.g. *Phytophthora infestans*) for which no *in vitro* activity has been demonstrated, suggest an indirect mode of action. Certain antibiotics inhibit the formation of galls of plants caused by *Agrobacterium tumefaciens*. Klemmer, Riker and Allen have demonstrated that the antibiotics were toxic to gall cells but non-toxic to normal healthy cells. By using resistant strains of the pathogens they were able to show that the effect was due to the susceptibility of the pathogen. Modifications of host metabolism may result in a changed host-parasite relationship, but there is very little evidence to support such a hypothesis. Other modes of action such as neutralization of toxins produced by the pathogen, the accumulation and unequal distribution of antibiotics within the plant and the conversion into substances of greater activity have been suggested. In the absence of any substantial data, it can only be assumed that the response to antibiotic treatment is the result of a combination of some or all of these factors.

There is no doubt that the use of antibiotics for the control of plant diseases is relatively expensive but in some cases they are far more efficient than most of the conventional fungicides and bactericides available at present. But the future of this new method of plant therapy will depend on the cost and not entirely on its efficacy. The cost of using these compounds should give a fair return to the grower. Antibiotics are now used on crops of higher-per-acre value e.g. fruits and vegetables, or when control requires small quantities of spray materials as in seed beds, and diseases like fire-blight of apples involving flower infection. The antibiotics now used for agricultural purposes are all crude forms of those produced primarily for medical purposes. The cost of these products are naturally high. In the U.S.A., some of the leading pharmaceutical companies have planned programmes specifically designed to develop antibiotics for agricultural use. The use of antibiotics in plant disease control may, therefore, be expected to be more widespread as and when crude antibiotics are available at competitive prices.

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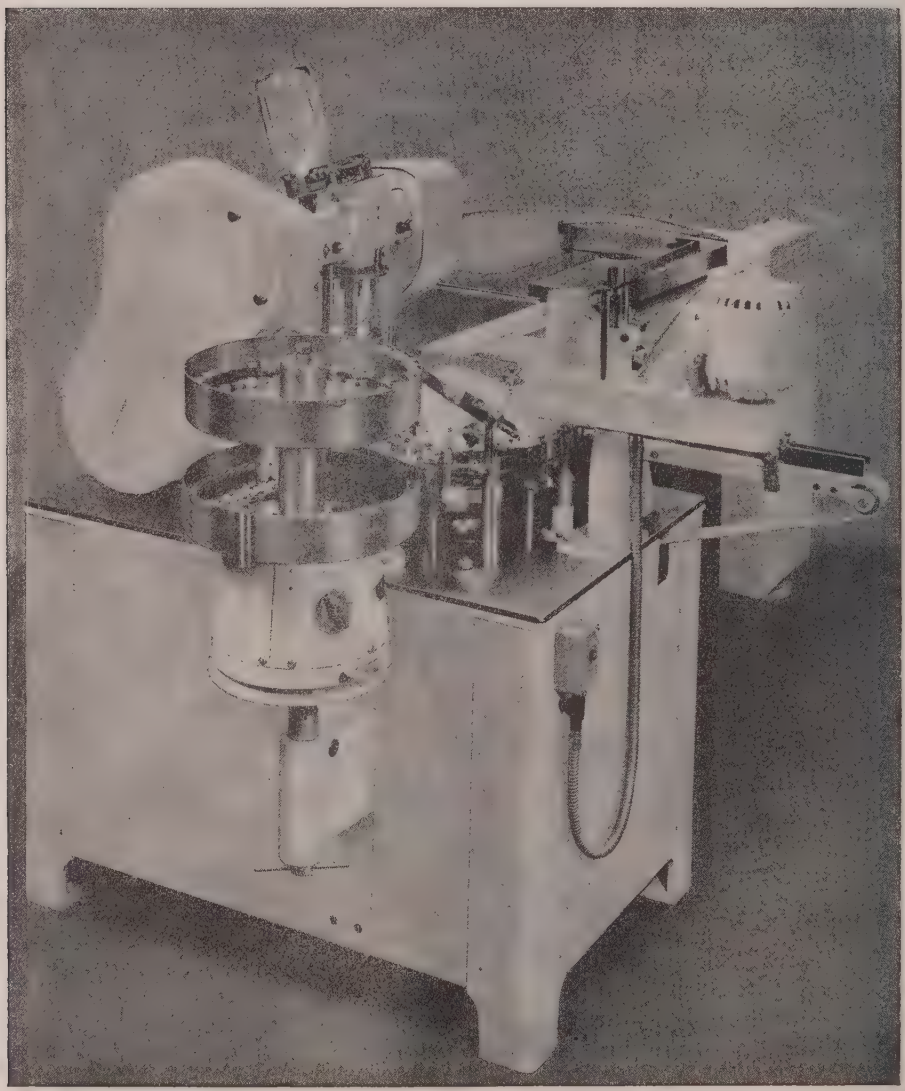

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Tissue Culture

B. K. BHUYAN, Ph.D. (Wis.)

TISSUE culture is the method whereby cells of plants or animals are taken out of the body and grown in suitable nutrient media so they can be studied as functioning individuals under controlled conditions. Unlike microorganisms (bacteria, yeast and fungi) the cells of higher animals live in a constant internal environment, and a state of symbiotic relationship exists between groups of cells. The development of a constant internal environment by higher animals in the course of evolution enabled the cells, sheltered from the changes in the outside world, to specialize in the performance of their functions. At the same time, however, the cells lost some of their synthetic abilities and became relatively dependent on other groups of cells. This mutual dependence is clearly responsible for the main difficulty in making cultures of animal cells away from their normal position. Therefore, unless the cells are cultivated *in vitro* in a surrounding very similar to their original environment, they languish and die. An intimate knowledge of the nutritional requirement of the cells and an understanding of their physicochemical environment are prerequisites for successful tissue culture.

Tissue Culture Nutrition

In the whole animal the cells of the tissues receive their nutrients through extracellular body fluids. Logically, therefore, early tissue culture media were prepared from lymph, plasma and tissue extracts, particularly chick embryo extract. A typical medium, for example, contained 20 per cent embryo extract, 40 per cent horse serum, and 40 per cent salts solution. The salt solution was designed to approximate the salts composition of the body fluids, provide buffers to maintain the pH at the physiological range (pH 7.0-7.8) and to maintain the osmotic pressure at the same level as the body fluids. Although such a natural medium permitted the long-term cultivation of a variety of cells, their complexity and variability made it nearly impossible to identify individual growth factor requirements. Better knowledge of the nutritional requirements of animals has made it possible to develop suitable synthetic and semi-synthetic media. The semi-synthetic medium of Eagle, for example, containing 12 amino acids, 9 B-vitamins, glutamine, glucose, salts and supplemented with dialyzed serum, was used to study the nutritional requirements of animal cells. It was possible to create specific amino acid, vitamin or mineral deficiency by excluding that particular component from the medium. Thus it was found that the tissues, *in vitro*, needed both phenylalanine and tyrosine, and also both methionine and cysteine. In contrast, the whole animal can convert phenylalanine and methionine to tyrosine and cysteine respectively and supply its needs for these two amino acids. L- and D-methionine were equally effective for tissue *in vitro* whereas only the L-isomer was active *in vivo*.

Culture Methods

Harrison, in 1907, first successfully maintained frog's nerve tissue alive and active in a hanging drop of frog's lymph. It was believed for a long time that a supporting framework was needed for the growth of the tissues. The tissue was grown in contact with a solid surface

(plasma clot, cellophane or glass) with stationary supernatant layer of fluid nutrient phase. Later developments included the "roller tube" where better interchange of metabolites between the cells (clinging to the glass surface of the test tube) and the fluid medium was obtained by gentle rotation of the tube. Owens and Gey in 1953 were able to propagate cells suspended in liquid medium under constant agitation. Since then cells have been grown as suspensions in vessels ranging from shaken flasks to 20-litre fermentors. As a result the large scale methods employed in the fermentation industries may eventually be applied to mammalian cell cultures.

Isolation of Pure Cell Strains

One of the major advances in cell cultivation methods was the demonstration by Sanford and co-workers in 1948, that strains of tissue cells could be derived through single-cell isolation like bacterial strains. Unlike bacteria, single cells isolated from the tissue were unable to grow when placed in a large volume of medium. However, isolated single cells could grow when placed in a small volume of medium, already preconditioned by the growth of the same tissue, contained in a capillary tube. At present stock cultures of pure strains of normal and malignant cells are isolated in this way.

Tissue culture has become an ideal tool for various disciplines. The following discussion will indicate but a few of them.

Application in Virus Research

The fact that viruses can grow on susceptible animal cells in tissue culture has dispensed with the use of animals for virus assay and for preparation of several viral vaccines. Smallpox, yellow fever and polio vaccines are prepared from viruses grown on susceptible cells. Besides being cheaper than the vaccine produced with the use of whole animals and more easily reproducible, this method results in a vaccine containing less extraneous proteins.

Screening of antiviral antibiotics and other agents has been made simpler, cheaper and faster by the use of tissue culture methods. The susceptible cells, grown *in vitro*, are infected with virus and then covered with agar. Filter paper discs soaked with the agent to be tested are placed on the surface of the agar. An agent causing the destruction of the virus will allow growth of the susceptible cells around the filter paper disc. However, the correlation between the antiviral activity *in vitro* and that *in vivo* is not very good. Therefore, this method can only be used as a primary screen to be followed by animal assay of the antiviral agents selected by the *in vitro* method.

Application in Cancer Research

Tissue culture methods have been used extensively in cancer research and tumour cells are among the most successfully cultured cell strains. Cultured tumour cells retain their malignancy as tested by grafting into a host animal. They can, therefore, be used to get information about the difference in biochemical behaviour of cancer cells from normal cells in order to explore ways and means of destroying them without simultaneous damage to normal cells.

Normal cells after serial cultivation in tissue culture show a great deal of similarity in histological appearance to malignant cells. This apparent transformation into malignant type under conditions which favour very rapid growth as in tissue culture, has aroused considerable interest. Several lines of investigation are being conducted to differentiate normal cells from malignant ones, grown in tissue culture. Foley separated normal from malignant cells by transplanting them into the cheek pouches of the golden hamster. Hamster accepts heterotransplants of malignant tissue but rejects heterotransplants of normal tissue. Thus it was found that when less than one million cells from normal tissue were transplanted they failed to

survive and grow. However, when only ten cells to one thousand cells of cancerous tissue were transplanted to the cheek pouch they grew. On this system a so-called "normal cell" strain D-189 behaved as a normal cell. However, on a system where the criterion was based on the ability to invade host tissue (cancerous cells are highly invasive) strain D-189 behaved like a cancerous cell. This indicates the great confusion existing regarding the normalcy of the so-called "normal cell".

An efficient method of screening anti-carcinogenic compounds should be rapid, inexpensive, should identify all potential anticarcinogenic compounds and not burden the investigator with false positive compounds. For the present, any new screening device must be compared with the slow and expensive animal tests lacking in correlation between activity in experimental tumours and in human cancer. Theoretically cancerous tissue grown *in vitro* could be used in the same manner as different bacteria are used in the screening of antibacterial antibiotics. The screening method is simply a measurement of the toxicity of a given agent to cancerous and normal cells. The cells are grown on tissue culture medium either in roller tubes or as suspensions in shaken flasks. The cultures are then incubated with a series of concentration of agents to be tested. Controls, to which the drug is not added, are included in each trial. After incubation the culture is examined either for cellular damage (assessed on the basis of granularity, disintegration, rounding up) or inhibition of cell multiplication. It was found that 80 per cent of the compounds which were active against tumours in mice were cytotoxic to cencerous cells grown in tissue culture. Of the compounds reported tumour negative (by mice assay) only 21 per cent were judged tumour positive by the tissue culture system. Thus the latter system did not burden the investigator with too many false positives. However, the majority of the anticarcinogenic compounds were also found to be cytotoxic to normal mammalian cells in tissue culture. This indicated that either the selective antitumour activity exhibited by many of these compounds *in vivo* was due to host imposed differences not obtained *in vitro* or due to the fact that the normal cells became malignant in the process of tissue culture. It is now evident that the tissue culture method is suitable for large scale use with considerable economy of time and effort as compared with primary screening in experimental animal tumours. The best use of experimental tumour systems may lie in the secondary and detailed study of those active compounds turned up by the *in vitro* primary screening.

Other Applications

Certain pathogenic fungi, such as *Histoplasma capsulatum* have two phases of growth—the pathogenic phase and the saprophytic phase. It is imperative in this case that the antifungal agents be tested against the pathogenic phase. It was found that fungi could be converted to their pathogenic phase by serial transfer in tissue culture media containing HeLa (a malignant cell of human origin) cells. After complete conversion to the pathogenic phase, these micro-organisms, suspended in a HeLa maintenance medium, have been used in the testing of antifungal agents.

Tissue culture methods have also been used in the study of the activity of drugs including antibiotics against tubercle bacilli growing in cells of human origin.

Usually tissues of members of the same species do not survive when transplanted from one individual to another (*i.e.* homografts) since the host responds by forming antibody substances to destroy the grafted tissue. Naturally, it would be a great boon if homografts could be used to replace damaged or diseased organs. An effort was recently made to acclamatize host and homograft by growing the necessary tissue in a medium to which serum of the prospective host had been added. Thyroid

glands of infantile rats have been explanted for periods of 3 to 4 months, and then grafted into adult rats from which the thyroid had been removed. There was evidence of an increased survival period, and uptake of radioactive I^{131} by the thyroids, indicating functioning of the glands. As an attempt to meet the demand for skin grafts, skin epithelial cells have been cultured in media to which different ingredients had been added to reduce antigenicity. Thereafter the cultures were preserved in frozen glycerol. However, there are many practical difficulties to overcome before such massive cultures can be used successfully as grafts.

Tissue culture methods have been extensively applied to the study of differentiation of normal tissue. A fully differentiated cell is one which has the necessary structure, —mechanical, physical and chemical— to enable it to function in a particular way. In course of the development of the embryo the organ rudiments become self-differentiating, that is they do not need the influence of any outside factors to allow normal development. The eye rudiment, for example, was removed from chick embryos of 66 hours incubation and explanted on the surface of solid coagula of plasma and embryo extracts. In such circumstances, the eye rudiment developed progressively in a normal manner to form the structures of a typical retina, pigment layer, rods and cones and lens of the eye. Similar developments of many other organ rudiments, like ovary, testis, and endocrine glands have been followed in tissue culture.

The enzymatic constitution of cell structures has not as yet been extensively investigated. All the enzymes of the citric acid cycle have been found in HeLa cell cultures. Transamination activity and a variety of enzymes related to nucleic acid metabolism have been reported. However, the general lack of precise information on the overall metabolic activity of tissue cultures is evident.

Conclusion

Tissue culture methods have travelled a long way from the early days when tissues were grown on hanging drops of lymph, to the present time when they can be cultivated in completely defined medium as cell suspensions in shaken flasks or 20-litre fermentors. Considerable controversy exists as to whether the so-called "normal cells" are converted to malignant cells under the conditions of rapid cell cultivation in tissue culture. Tissue culture methods are eminently suitable as primary screens for anticarcinogenic and antiviral compounds, and they are finding increasing application in the production of viral vaccines. Experimental embryology has reaped enormous harvests from tissue culture methods. However, the enzymatic constitution of cell cultures still remains a relatively unexplored field. The study of isolated cells and tissue *in vitro* has altered the concept of the cell. It is no longer simply a sub-division of living matter but is a living and changing unit constantly varying in response to its environment. Tissue culture at present is a field expanding at a fast pace and great developments are hoped for in the future.

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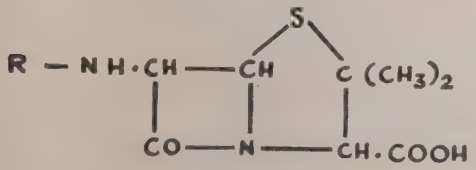
Penicillin in its New Perspective

S. K. DAS GUPTA, Ph.D.

NEARLY two decades ago the Oxford group of researchers led by Florey and Chain reported, for the first time, the isolation of penicillin, apparently in an impure form. This was a great advance indeed, for the earlier unsuccessful attempts of Raistrick to isolate the extremely unstable principle hardly gave any impression of penicillin's great future in chemotherapy. Considerable progress has since then been made in the technology of penicillin production, and penicillin has come to stay as an antibiotic of immense value because of its exceedingly low toxicity and comparative cheapness.

The elusive and labile nature of the penicillin molecule coupled with its high therapeutic value was both fascinating as well as challenging to the organic chemist to probe into its constitution. Investigations established penicillin obtained by fermentation to be a mixture of closely related compounds having in common a fused thiazolidine-β-lactam nucleus (I) and differing in the nature of the side chain.



(I)

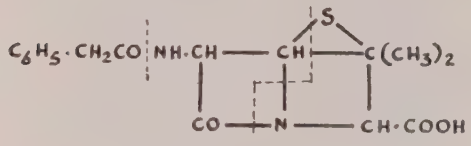
Ia, R = H (6-Aminopenicillanic acid)
Ib, R = $CH_3.CH=CH.CH_2.CH_2.CO$ (Penicillin F)
Ic, R = $C_6H_5CH_2CO$ (Penicillin G)
Id, R = $CH_3.CH_2.CH_2.CH_2.CH_2.CH_2.CO$ (Penicillin K)
Ie, R = $CH_2=CH.CH_2.S.CH_2CO$. (Penicillin O)

The formation of the side chains is dependent on the chemical environment of the mould. The differences in the side chain characterise the penicillins not only in their stability in acid condition but also in their biological activity. Benzylpenicillin (penicillin G)—the most widely used member of the group—was obtained as the major constituent of the mixture of penicillins by the addition of cornsteep liquor to the culture medium. In the early days of penicillin production n-heptylpenicillin (penicillin K) was the most frequent and major contaminant. The present method of adding the precursor phenylacetic acid at intervals to the culture medium avoids the formation of this penicillin to a large extent.

As a consequence of the determination of the structure of the penicillin molecule, attempts have been made to prepare derivatives having better therapeutic properties. Among the series of esters the diethylaminoethyl ester of benzylpenicillin is of value in the treatment of pulmonary infections, as it preferentially concentrates in the lungs. Other modifications have been effected mostly on p-hydroxybenzylpenicillin. However, none of these compounds seem to be better than the common penicillin G in therapeutic efficacy. Benzylpenicillin sulphone, for instance, has only one-tenth the activity of penicillin G.

The fact that differences in side chain are responsible for the differences in properties of various penicillins led biochemists to introduce modifications in the penicillin molecule by changing the conditions of fermentation. During the early days of

research on the total synthesis of penicillin it became apparent to the Eli Lilly group of workers that complete success of the synthetic programme was by no means assured due to the complicated stereochemistry of the penicillin molecule. They advanced a biogenetic scheme for penicillin which was taken to be composed of three moieties (II) corresponding to phenylacetic acid, aminomalonsemialdehyde, and penicillamine.



(II)

Research was then directed to determine whether penicillin degradation products, possible metabolic intermediates or related compounds would be capable of stimulating penicillin biosynthesis by acting as precursors. It was found that the mould *P. chrysogenum* was capable of metabolising a large variety of precursors containing new acyl groups leading to the biosynthesis of hundreds of new penicillins in good yields. Thus phenoxymethylpenicillin (penicillin V) which is acid stable and of value in oral therapy, was first obtained by the Lilly research group in 1948 but its therapeutic usefulness was not discovered until much later. Similarly, allylmercaptomethylpenicillin (penicillin O) and *n*-butylmercaptomethylpenicillin (penicillin BT) are of proved therapeutic value. Patients sensitive to penicillin G tolerate penicillin O without allergic reactions.

Cephalosporin N or synnematin B, produced by two different moulds, has been shown to be a new penicillin with a side chain derived from D-α-aminoadipic acid. Florey suggested that the new penicillin be named penicillin N or aminocarboxybutylpenicillin although *P. chrysogenum* does not incorporate the amino acid into the penicillin molecule. The antibacterial spectrum of this penicillin stretches beyond the gram positive organisms to include the typhoid *Salmonella* of the gram negative group, and the specificity of its action is due to the amino acid entity in the side chain. *p*-Aminobenzylpenicillin is another example of a new side chain conferring new biological properties to penicillin. This compound maintains effective penicillin blood levels for a longer time than penicillin G and is also active against typhoid bacilli *in vitro*. These examples point to the possibility of incorporation of basic functions in the side chain to give penicillins which may be active against organisms such as gram negative bacteria and viruses which are not susceptible to common penicillins.

The capacity of *P. chrysogenum* to metabolise precursors is limited although many precursors could be incorporated by the mould. This fact has until recently been a major handicap in the programme of biosynthesis of penicillins. The apparent success of producing new penicillins by fermentation was not, however, a deterrent to attempts at the total synthesis of the antibiotic. Sheehan, after nearly ten years of research, reported in 1956 the total synthesis of methyl-DL-benzylpenicillinate sulphone and DL-penicillin V. This success gave rise to expectations of the availability of new synthetic penicillins which may be inaccessible via biosynthetic routes. Here again, the number of steps involved and proper stereochemical controls at every stage in the synthesis were the main difficulties. It seemed that complete success in producing new penicillins with desired side chain depended on getting the fused thiazolidine-β-lactam structure (Ia) in the right stereoisomeric form and in good yields for which microbiological production appeared to be the preferred and practicable method. From biogenetic considerations Arnstein and other workers believe that during penicillin production by fermentation the formation of (Ia) takes place first followed by acylation effected through

specific enzyme systems. The Japanese workers Sakaguchi and Murao (1950) and Kato (1953) reported that (Ia), named penicin by them, was formed in cultures of *P. chrysogenum* in the absence of side chain precursors or by the hydrolysis of benzylpenicillin under the influence of an acylase. These claims, however, remain unconfirmed.

Recently Batchelor, Doyle, Nayler and Rolinson of the Beecham Research Laboratories in U.K., continuing their work on *p*-aminobenzylpenicillin in association with Prof. Chain and colleagues, reported isolation of 6-aminopenicillanic acid (Ia) in crystalline form from penicillin fermentations carried out with a *P. chrysogenum W.* 51.20 isolate in the absence of added side chain precursors. Their claim has been established beyond doubt by the synthetic conversion of (Ia) to penicillin G as well as penicillin V. This discovery is a remarkable achievement pregnant with far-reaching potentialities. The new development offers promise of solutions for some of the urgent problems in penicillin therapy, as for instance, new approaches to combat the widespread resistant strains of staphylococci, and penicillins effective against gram negative bacteria, viruses, etc., might be possible. With the isolation of 6-aminopenicillanic acid in quantity hundreds of new penicillins, almost tailor made to cover a wider range of diseases are likely to be available on a commercial scale. Thus a partnership between microbiology and organic chemistry holds out promise of a bright future for penicillin by widening the frontiers of its therapeutic activity.

Research Notes

PENICILLIN V (PHENOXYMETHYL PENICILLIN) SALT OF N-BENZYL-3β-CHOLESTERYLAMINE

A few salts of penicillin V, such as N, N-dibenzylethylenediamine dipenicillin V[1] and quinine penicillin V[2] have been reported in literature. In an earlier paper[3, 4] we reported the preparation of penicillin G salts of N-benzyl-3β-cholesterylamine and N-benzyl-6β-amino-3:5-cyclocholestane and the effect on biological activity with the variation of the structure of the parent amine. In the present work attempts were made to prepare penicillin V salts with the above bases. N-benzyl-3β-cholesterylamine alone formed penicillin V salt although both the bases formed salts with penicillin G. There was no salt formation when N-benzyl-6β-amino-3 : 5-cyclocholestane hydrochloride was reacted with potassium penicillin V in different solvents like methanol, aqueous methanol, moist ether and mixture of methanol and chloroform, and also when the free base was reacted with penicillin V acid in acetone.

The hydrochloride of the base $C_{35}H_{55}N$ (prepared by reaction of N-benzyl-3β-cholesterylamine with formic acid and formaldehyde) was reacted with potassium penicillin V in aqueous methanol and mixture of methanol and chloroform but salt formation did not take place. This amine also did not form salt with penicillin G.[4]

N-benzyl-3β-cholesterylamine penicillin V is tasteless and stable to acid pH. On keeping it at 37°C and pH 4.5 for 2 hours the activity was 676 u/mg as compared to the original potency of 714 u/mg.

EXPERIMENTAL

Preparation of N-benzyl-3β-cholesterylamine Penicillin V

To a solution of 3.0 g. (0.00586 mole) of N-benzyl-3β-cholesterylamine in 400 ml. of 90 per cent aqueous methanol, 4.56 g. (0.0117 mole) of potassium penicillin V was added gradually while the mixture was vigorously stirred at 30°C. White crystals appeared after three and half hours of continuous stirring. After keeping overnight at 5°C, the crystals were filtered off and washed with cold methanol to give 4.116 g. of the penicillin salt, m.p. 148-50°C. On concentrating the mother liquor under reduced pressure, 0.458 g. of additional penicillin salt was obtained. The salt was recrystallized from aqueous methanol. There was no change in m.p. The overall yield of the penicillin salt was 94 per cent of the theoretical amount on the basis of the base hydrochloride used.

The potency of the salt by chemical assay was 712 u/mg. and 714 u/mg by bioassay as compared to the theoretical potency of 718 u/mg.

$(\alpha)_D^{24}$, +5.3° (35.35 mg. dissolved in 1 ml. of chloroform, α, 0.09° ; 1, 0.5 dm.)

For analysis the sample was dried at 65°C under 0.5 mm. pressure for 72 hours. Calculated for $C_{50}H_{71}N_3O_5S$: C 72.69, H 8.66, N 5.1 ; found C 72.56, H 8.60, N 5.3.

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PRELIMINARY NOTE ON THE EFFECT OF ANTIBIOTICS ON PHOTO-SYNTHESIS IN *HYDRILLA VERTICILLATA*

Investigations on the effects of antibiotics on higher plants have not been extensive, though antibiotics have been used in controlling phytopathogens. Brian[1] has recently reviewed the subject. While most of the investigations deal with the stimulation and inhibition of growth by antibiotics, at least one has dealt with photosynthesis. Thus, Havinga *et al* observed increased carbon dioxide fixation by *Scenedesmus obliquus* on stimulation with oxtetracycline and chlortetracycline. Inhibitory effect on pigment formation by streptomycin has been observed by a number of investigators quoted in the review.[1]

The mechanism of photosynthesis is known to be so delicately balanced that addition of substances in very minute doses (of the order of 10^{-5} moles per litre) are sufficient to produce a significant response. Moreover, aquatic plants are ideally suited for such experiments because of their ability to absorb substances from all along their body surface and also because of the comparative ease with which photosynthesis can be evaluated by a measurement of the oxygen liberated.

METERIAL AND METHOD

Hydrilla verticillata Presl. Bot. was grown in glass jars in the laboratory. The plants were radiated for successive intervals of five minutes of light alternating with five minutes of darkness. The light source was a 1,000 watt Phillips filament bulb fitted with a search-light type assembly and placed at a distance of 24 in. from the plant. The temperature was regulated at $33^0 + 0.5^0$C. The amount of oxygen evolved was measured in an Audus microburette.[3] The medium used was 1/4 strength Hoagland's solution with 0.5 per cent potassium bicarbonate.

The plant twig, 5 in. in length, was allowed to photosynthesise for 10 minutes in continuous illumination. The gas collected during this period was neglected since it contained a large proportion of nitrogen from the intercellular spaces. Three successive readings were then taken during five minute illumination periods. Usually they coincide and the mean taken as starting value for the oxygen produced by the twig under the conditions specified above. The antibiotic was then added so as to make up a definite concentration in p.p.m. The gas evolved during further successive intervals of illimination was recorded as a percentage of the starting value. Thus, the percentage inhibition or stimulation produced by a known concentration of the added antibiotic could be read off directly from the table. A duplicate series was run at the same time as a control for the distilled water or any other diluting fluid used. The results obtained are given in brief in Table below.

Antibiotic	Dilution	Response
Penicillin G sodium	100 p.p.m.	35-40% inhibition
	0.1 p.p.m. and lower dilutions	No stimulation
Streptomycin sulphate	10 p.p.m.	12-20% inhibition
	0.01 p.p.m.	8-12% stimulation
Citrinin	10 p.p.m.	35-40% inhibition
	0.001 p.p.m.	35-37% stimulation

From these preliminary experiments it is apparent that the responses at different dilutions are fairly constant. The actual

amount of response varies with specimen within wide limits. It has also been noted that the percentage of stimulation depends on the growth vigour of the *Hydrilla* shoot tested. Those plant twigs which produce comparatively very little oxygen as their starting value show a much higher degree of stimulation than normal plants.

ACKNOWLEDGMENT

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A DIRECT METHOD FOR ASSAY OF PENICILLIN IN FERMENTED BROTH SAMPLES. Part II*. DETERMINATION OF PENICILLIN V (PHENOXYMETHYL PENICILLIN)

In an earlier communication[1] we reported a direct method for the assay of penicillin G in fermentation broth samples. A similar method for the assay of penicillin V is reported here.

In penicillin V fermentations, other penicillins—mainly penicillin G—are produced along with penicillin V, as evident from extraction efficiencies and biochromatograms. This is due to the presence of phenylalanine, a precursor for penicillin G, in corn-steep liquor used in the fermentation medium. We have studied the applicability of the direct method[1] for the differential assay of penicillin V. The results compare well with those obtained by standard extraction methods and also agree with subsequent crystallization recoveries.

The quantity of iodine required for reacting with the alkali decomposition product (penicilloic acid) of penicillin V and penicillin G are not the same. Goodey *et al*[2] reported an absorption of 2.46 ml. of 0.01N iodine per mg. of penicillin V acid after penicillinase inactivation, while we obtained 2.665 ml. of 0.01N iodine per mg. of penicillin V after alkali inactivation. In a number of determinations, the penicillin factor (described in the earlier paper[1]) for the same strength of sodium thiosulphate (0.01N) differed by 22 units and corresponds to 9.337 atoms of iodine per mole of penicillin V (acid) as against 8.4 atoms for penicillin G (acid) (Table I).

TABLE I

Penicillin G and V Factors at pH 2 (FDA method)

S. No.	Pen. G	Pen. V	Diff.
1	660	638	22
2	655	632	23
3	666	643	23
4	671	650	21
5	658	636	22
6	666	644	22

This difference is high even allowing for the higher molecular weight of penicillin V.

The values obtained for pure crystalline samples of potassium penicillin V were higher and more than the theoretical, when the factor using standard penicillin G was used for calculation[3]. A pure sample of potassium penicillin V, analysing for 100 per cent penicillin V content[4] was used for calculation of the factor.

TABLE II

Potassium Penicillin G Crystalline Samples

Sample No.	Unitage calculated with G factor	Unitage calculated with V factor
A	1,556	1,530
B	1,552	1,526
C	1,556	1,530

* *For* Part I *see* Reference 1.

Variation of the factor for penicillin G with the pH of titration was reported earlier.[1] A similar variation in the factor with pH was also noticed for penicillin V (Table III). The difference in values at pH 2 and 4.5 in the case of penicillin V is almost the same as with penicillin G.

TABLE III

Penicillin V Factors at pH 2 and 4.5

S. No.	Factor at pH		Difference
	2	4.5	
1	648	728	80
2	649	729	80
3	609	688	79
4	616	698	82
5	608	690	82
6	648	728	80

The differential assay for penicillins G and V is based on their relative stability at acid pH. A detailed study was made with pure solutions of penicillins G and V and their mixtures with respect to their stability at acid pH with different concentrations of acid. While there was a rapid rate of inactivation of penicillin G at acid pH, it was not complete even after 1 hour if the solution was brought to pH 2 with acid. However, if the acid concentration was increased the decomposition of penicillin G was almost complete within 30 mins. The stability of different concentrations of penicillin G (as it occurs in penicillin V fermentation) with higher acid concentrations is given in Table V.

TABLE IV

Stability of Penicillin G and Penicillin V Solutions at pH 2

Time in minutes	Penicillin G soln. (Initial unitage 2,170 u/ml)	Penicillin V soln. (Initial unitage 2,180 u/ml)
	u/ml	u/ml
15	1,096	2,100
30	770	2,050
45	415	1,940
60	400	1,690
90	265	1,300

TABLE V

(1·5 ml. phosphoric acid, 30 per cent W/V for 25 ml. of soln.)

S. No.	Time in minutes	Penicilin G concentrations u/ml.		
1	0	500	250	200
2	10	200	150	80
3	20	150	66	50
4	30	Negligible	Negligible	Negligible

With the same concentration of acid, penicillin V lost only 15 per cent of the activity (Table VI) and, therefore, with necessary corrections, the penicillin V content could be evaluated.

TABLE VI

Penicillin V Stability

Penicillin V concentration, 2,150 u/ml.
Phosphoric acid concentration, 30% W/V

Time in minutes	0.5 ml. of acid for 25 ml. soln.	1.0 ml. of acid for 25 ml. soln.	1.5 ml. acid for 25 ml. soln.
15	2,055	1,935	1,885
30	2,015	1,920	1,830*
45	1,935	1,790	1,665
60	1,625	1,545	1,430

*15 per cent of activity lost.

Another important factor to be taken into account is the variation in the blank values due to the decomposition products of penicillin produced by acid treatment. An appropriate correction factor for this was worked out as follows. Pure penicillin V solutions were put for acid decomposition for 30 mins. ; the blank was then carried out at pH 4.5 (after neutralizing the excess acid) and the difference in blank titrations before and after acid treatment noted. This, naturally, varied with the penicillin V unitage and the fall in the blank was compensated for in the actual determinations

with broth fluids. This is equivalent to 0.7-0.9 ml. of 0.01N sodium thiosulphate in the range 1,500 to 3,000 u/ml.

On the basis of the above results the procedure for the estimation of penicillin V in broth samples has been worked out as follows.

PROCEDURE

To the broth filtrate (25 ml.) was added phosphoric acid (30 per cent W/V : 1.5 ml.), the solution stirred and set aside for 30 mins. The solution (2 ml.) was pipetted out into two test tubes, for blank and test, neutralized to pH 6-7 with 1N sodium hydroxide. To the blank was added buffer (5 ml.) pH 4.5 (potassium acetate-acetic acid), and 0.01N iodine (10 ml.) and after 15 mins. titrated against 0.01N sodium thiosulphate. To the test was added 1N sodium hydroxide (2 ml.) After 15 mins. the solution was acidified (pH 2-3) with 1.1N hydrochloric acid and buffer (5 ml.), followed by addition of 0.01N iodine (10 ml.) After 15 mins. the excess of iodine was titrated as usual. The blank correction was applied to the actual blank obtained by a separate determination. The penicillin V content in the broth was calculated by the formula:

$$\frac{(B_{corr} - T}{2} \cdot Fv. \frac{100}{85}$$

where B_{corr} is the corrected blank, T the test titre, Fv the penicillin V factor with a working standard (potassium penicillin V) at pH 4.5 and 100/85 the correction necessary to compensate for the loss of penicillin V itself under the conditions of test.

The validity of the method was checked by increment analysis using penicillin G broth at a unitage of about 500 u/ml. (the concentration having been chosen on the basis of our experience with production fermentors where the acid decomposible penicillins are between 300 and 500 u/ml.) The results, as may be seen from Table VII, are in close agreement.

In our experience over a number of batches the values obtained by this modified direct method agree closely with those obtained with other process samples in extraction and crystallization and the overall efficiencies.

TABLE VII

Increment Analysis

S. No.	Pen. G u/ml.	Pen. V added u/ml.	Pen V obtained u/ml.
1	500	1,520	1,550
2	500	2,000	2,050
3	500	2,510	2,480
4	430	2,080	2,120
5	300	2,060	2,120
6	380	2,060	2,100
7	300	2,390	2,420
8	300	1,500	1,500

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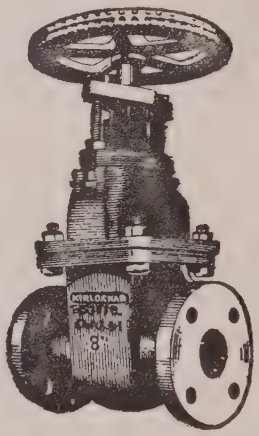

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Antibiotics Information

Trends in Antibiotics Production

(IV)[1] ASIA & THE FAR EAST

Japan

Within the last decade Japan has built up a vigorous basic drug industry which in 1956 had a record production valued at £100 million. Local manufacture is encouraged, and after the second World War, a number of technical assistance contracts on royalty basis were concluded with foreign manufacturers, mainly American. As recently as 1956 Pfizer International set up a fermentation plant in Kobe, with tetracycline as one of its products, as a joint operation with local firms. In the early days of the antibiotic industry in the country quite a large number of local firms came forward to set up manufacturing units. Today there are a dozen leading firms producing antibiotics. Of the four streptomycin production units two have technical collaboration with Mercks of U.S.A.

Prior to 1949, only amorphous penicillin was produced, but now most of the useful antibiotics like penicillin, streptomycin, dihydrostreptomycin, chloramphenicol and the tetracyclines are manufactured on a large scale. Japan's pyrimidine penicillin is comparable to procaine penicillin in its biological action. On a small scale, the following antibiotics are produced : Colistin (for whooping cough), aureothricin (for mycoses), leucomycin (a possible substitute for erythromycin), trichomycin (for *Trichomonas vaginalis*), neomycin and the antitumour antibiotics sarkomycin and carzinophilin. Penicillin output registered a steady increase but seems to have levelled off at about 35 m.m.u. in the last three years. In 1956, the value of the output of antitubercular drugs like streptomycin, dihydrostreptomycin and P.A.S. was up by 22 per cent (4.13 mill. yen) over that in 1955. Similar rise in production was reported for chloramphenicol and tetracycline. Surplus penicillin was exported to S. E. Asian countries and during Japan's five year plan period (1954-58) the export was to have been stepped up substantially.

Japan—Five-Year Plan (1954-58) Estimates

Year	Production capacity		Domestic demand		Possible export	
	P. m.m.u.	S. tons	P. m.m.u.	S. tons	P. m.m.u	S. tons
1954 ..	50	36	25	24	25	32
1956 ..	60	41	30	24	30	17
1958 ..	60	41	30	24	30	17

P = Penicillin S = Streptomycin

Japan—Production of Penicillin and Streptomycin

Year	Penicillin (Finished products) in 100 mill. u.	Strepto-mycin kg.	Dihydro-streptomycin kg.
1946 ..	0.46		
1947 ..	136.00		
1948 ..	2,968		
1949 ..	17,959		
1950 ..	74,955		
1951 ..	146,696	2,063	138
1952 ..	237,049	1,056	5,090
1953 ..	495,683	321	28,557
1954 ..	477,797	1,954	39,035
1955 ..	487,148	25,321	22,295
1956 ..	344,200	28,066	37,575
1957 ..	360,000 (approx.)		

(1) Part (I) covering U.S.A., Latin America and Canada appeared in V. 1, p. 45, Part (II) covering U. K. and Western Europe on p. 71, and Part (III) covering Eastern Europe, Middle East and S. Africa on p. 96, of this *Bulletin*.

However, there is keen competition from products imported from the West, and indigenous production units have been or are being set up in a number of the Asian countries.

Production of other antibiotics in 1956 was : Trichomycin 1,429 bill. units, chloramphenicol 9,868 kg., chlortetracycline 1,308 kg., sarkomycin 376 kg.

JAPAN—Export of Antibiotics (in kg.)

	1955	1956
Penicillin	3,235	7,315
Streptomycin	5,212	20,648
Chlortetracycline ..	56,128	2,137

JAPAN—Import of Antibiotics

Product	1955 Quantity gm.	1955 Value in 1000 yen	1956 Quantity gm.	1956 Value in 1000 yen
Penicillin	459,452	54,808		
Streptomycin	64	21		
Chlortetracycline	5,695,200	61,635		
Antibiotics n.e.s.	10,259,794	732,703	219,560	46,244
Medicinals and Pharmaceuticals ..	299,339,000	1,793,267	173,855,000	540,932

American and European firms are now taking up production of some of Japan's new antibiotic specialities. The Fujisawa Pharmaceutical Co., has licensed the German firm Chemie Gruenenthal GmbH, Stolberg, to produce trichomycin. The German firm can sell the antibiotic in Federal and East Germany and export to Austria, Iran, Lebanon, Jordan and Saudi Arabia. Kanamycin, active against resistant staphylococci, is now marketed by the Bristol Laboratories, who have also taken up production of Mitomycin C, for experimental evaluation against cancer.

China

China's first antibiotic plant, which began penicillin production in 1952, was a converted old motor workshop, and the first antibiotic production unit built with Russian assistance went into operation in 1953. A decade ago there were few pharmaceutical enterprises in the country ; some local firms were making capsules and ampoules, but a majority of the modern drugs were imported. In the first five-year plan period (1953-57) Chinese pharmaceutical industry is reported to have made considerable progress. As many as 200 different drugs are now mass produced and of these penicillin, streptomycin, chlortetracycline, syntomycin (chloramphenicol), sulpha drugs and vitamins are important items. Oxytetracycline and other essential drugs have already passed the pilot plant stage and plants for their large scale manufacture are being established. The targets for the first plan were :

Antibiotic	Production in 1952	Target for 1957
Penicillin (Unit: 1,000 ampoules each containing 3 lakh I. U.)	153	29,000
Chloramphenicol (kg.) ..	—	6,000
Sulphonamides (1,000 kg.) ..	81	844

At the end of the first plan total production of drugs was nearly 5.75 times the output in 1952. Imports were reduced to about 10 per cent in 1957 as compared to those in 1952 with respect to the total

amount of drugs sold in the country. The number of fermentation units for penicillin

China : Pharmaceutical Imports
(in million$)

1953	..	..	3.3
1954	..	..	10.0
1955	..	..	6.7
1956	..	..	5.0

in 1957 was about three times what was available in 1953. For the first half of 1957 the Pharmaceutical Industry Administration Bureau of Mainland China announced an output of 9 m.m.u. of penicillin. The output of chloramphenicol in 1957 was nearly 18 tons compared to 6 tons in 1953. Cost of production and selling price of antibiotics decreased simultaneously, *e.g.* cost of production of chloramphenicol decreased 67.9 per cent compared to that in 1955, while that of penicillin in 1957 was only about 25 per cent of the cost in 1953. Factors helping the rapid growth of the Chinese pharmaceutical industry are reported to be the construction of over forty plants and workshops, effective use of technological progress and improvements in existing factories, and substitution of indigenous and cheaper raw materials for costlier imported ones. The quality and standards of Chinese drugs have also improved through better quality control and product development programmes. Concerted efforts are being made towards self sufficiency in basic drugs and a programme for doubling and even trebling the output of pharmaceuticals during the second plan period 1958-62, has been worked out. The estimated output of all antibiotics at the end of the second five-year plan (1958-62) is reported to be about 1,200 tons, the present capacity being over 100 tons. A number of new factories and workshops are to be established, and penicillin and streptomycin production to be stepped up respectively to 50,000 and 36,000 kg. annually. The economic outlay for 1958 provided for a total of 103,000 kg. of antibiotics, an increase of nearly 200 per cent over 1957 output. Capital investments in pharmaceutical production for 1958 was about £35 million. New plants include a factory for pharmaceuticals (including penicillin and streptomycin) at Taiyuan in North China set up with Soviet assistance; chlortetracycline is to come from No. 3 Pharmaceutical Works. The pharmaceutical industry in Sinkiang province is to be developed and among the new plants one is for chlortetracycline. The country's largest and most modern of the pharmaceutical works—the North China Pharmaceuticals Plant—the construction of which was begun in 1955, went into operation early in 1958. It has departments for antibiotics, starch, glass, etc. The plant was designed and constructed by Russian and East German engineers and scientists and Chinese technicians were also trained in those countries.

With the production of twenty times more antibiotics in 1958 than in 1955, China's requirements of penicillin and streptomycin are reported to be completely met by indigenous manufacture. The Shanghai pharmaceutical plant has started production of vitamin B_{12} from chlortetracycline production wastes. Daily output is said to be about 60-90 thousand ampoules, sufficient to meet home demands. A number of state farms are setting up small units to produce chlortetracycline, oxytetracycline and penicillin for use in veterinary medicine, and poultry and animal feeding. Considerable research is also reported in the field of antibiotics production and use. The recently discovered antibiotic actinomycin K has shown encouraging results in animal experiments in the treatment of cancer.

To meet the demands of the expanding pharmaceutical industry a special project institute with a team of project and engineering personnel has been set up, in addition to the designers and engineers in the indivi-

dual plants. Factories to manufacture equipment specially required by this industry are also being set up. The Soviet Union and other socialist countries have extended considerable assistance, financial and technical, in these developments.

With the rising production, China has also started intensive export drives, in Western Europe as well as in Asian countries. In Hong Kong, chlortetracycline (Gold Mycin) is reported to be marketed at prices one-third below those of the corresponding U.S. drug. South African imports of Chinese pharmaceuticals, vitamins and sulphas are considerable. The Chinese trade mission to Australia has booked orders worth several million dollars for pharmaceuticals and fine chemicals. Catalogues of Chinese fine chemicals and pharmaceuticals, offered at bargain prices, are flooding European chemical market. The quality of the Chinese materials is reported to be comparable to those put on the market by other countries.

Hong Kong

Merck and Co. of U.S.A., have recently established a penicillin packaging plant in Hong Kong. Antibiotics manufactured in U.S., will be repacked for local distribution and for shipment to other Asian countries. The plant costing about $333,000 would employ about 50 local residents. Capital investment and sales promotion would be by local companies now handling U.S. pharmaceuticals, but quality control and distribution are directed by the U.S. company, and its brand name will be on the repacked goods. The bulk shipment of antibiotics from U.S. for repacking to market size packages under favourable free enterprise conditions is expected to reduce production costs and help meet the competition in the Far East.

Philippines

A number of American firms have established branch offices and manufacturing units in the island. In the initial stages the industry was mostly confined to repacking imported bulk material, but the trend is towards actual local manufacture.

Pfizer International set up two plants in 1954 and operations began the following year. The company's activities are being expanded.

Also in 1954, Parke, Davis and Co., opened its first branch office and manufacturing laboratory near Manila to produce antibiotics, vitamins, etc., at an initial cost of $500,000.

Merck, Sharp and Dohme (Philippines) Inc., Manila, producing antibiotics, hormones, pharmaceuticals and rice premix for distribution throughout the island, is expanding its plant facilities.

Other American companies engaged in local manufacture of pharmaceuticals include Eli Lilly, E.R. Squibb, Wyeth International, Sterling Products International, Vick International, and Abbott Laboratories.

Australia

Australia has attracted a number of foreign firms to establish local manufacturing and sales units. A good portion of the country's pharmaceutical requirements are imported from the United Kingdom. In 1956-57 total drug imports were valued at £5.6 million of which 60 per cent came from U.K.

The Drug Houses of Australia Ltd. (D.H.A.) and the American Cyanamid Company have entered into an agreement by which the former will manufacture a number of Lederle drugs. Tablets and ointments are now produced in the laboratory at Rozelle. A special building costing £30,000 has been put up at Roseberg for encapsulation work using American Cyanamid's Accogel machine. D.H.A. engineers

and technicians designed the building in collaboration with Lederle experts; special equipment came from U.S.A. and U.K., but a major portion of the plant was constructed in Australia. The encapsulation unit is now mainly used for antibiotic preparation.

Merck, Sharp and Dohme (Australia) Pty. Ltd., are expanding their plant facilities at Fairfield, New South Wales.

Eli Lilly (Australia) Pty. Ltd., is to establish a £400,000 highly automatic ethical products plant at Ermington, N.S.W. The factory, commencing operation this year, will concentrate on production of tablets and ampoules for the Australian market. Export to other Asian countries will be considered at a later stage.

Pfizer International's manufacturing operations in Australia began in 1954. The company has purchased lands at Ermington near Sydney for a new plant (£A 300,000) for compounding and packaging antibiotics using basic material imported from U.S.

Abbott Laboratories (Australia) Pty. are planning a plant at Sydney to produce pharmaceuticals and fine chemicals but no fermentation products.

Upjohn Co. (Australia) Pty. Ltd., in temporary quarters in Sydney since 1956 have now an eleven acre site in Parramatta in Sydney's suburbs, for plant, manufacturing and warehousing facilities.

The Roussell Group of France which specializes in the manufacture of antibiotics, hormones and other drugs, is planning to establish a factory in Sydney.

The Swedish firm A.B. Astra, Södertralje, is setting up an antibiotics manufacturing unit—Astra Pharmaceuticals Pty. Ltd.—in Brisbane.

Other foreign companies with interests in Australia are the Commonwealth Laboratories, Glaxo Laboratories, and Monsanto Chemicals.

Pakistan

Pakistan's first penicillin plant, a collaborative effort between Pakistan Industrial Development Corporation and UNICEF, designed to produce 7 m.m.u. per year to meet the entire requirements of the country, is expected to go into production early next year. The installation of the plant at Daudkhel (West Pakistan) is nearing completion. UNICEF's contribution to the project by way of equipment, training of personnel etc. is of the order of $6 lakhs. At a later stage production may be increased to 10-12 m.m.u. per year. Some of the scientific and technical staff for this project were trained at Hindustan Antibiotics, Pimpri, India.

Ceylon

Glaxo Laboratories of U.K., and Dumex Ltd., of Holland are to produce antibiotics, other drugs and children's foods, in their factory now under construction in Ceylon.

Burma

On a contract entered in 1953 with the Burmese Government, Evans Medical Supplies of U.K., are to provide Burma with a complete pharmaceutical industry including pharmaceuticals, biologicals, yeast, alcohol, etc. Designated as the Burma Pharmaceutical Industry the company completed construction in about two years and went into operation in 1957, at Gyogon near Rangoon. Divided into six main buildings of which one is for pharmaceutical manufacture (including raw materials, finished goods storage, and quality control laboratories). This department has a production division, sterile products units, tabletting, "galenicals", and filling sections. The Burmese Government took over the management of the company in February this year.

Indonesia

Most of the drugs, specially antibiotics, are imported from U.S.A., U.K., Holland and Germany. The three manufacturing units existing in 1954 were producing common pharmaceuticals. In 1956, Carlo Erba S.p.A. of Milan in association with the Indonesian Ministry of Health commenced construction of a plant in Bandung for the manufacture of antibiotics. The cost of the plant is about 4 million rupiahs.

ANTIBIOTICS INDUSTRY IN INDIA

The Background

Against the background of India's medical tradition extending far back into the third millenium B.C., her pharmaceutical industry is barely a century old, and her antibiotics industry is just in its infancy. And yet the achievements even of the last three or four years are pointers to and justify the hopes of, self sufficiency in the local production of essential drugs in the next few years.

Pioneering efforts of K. T. Gajjar and B. D. Amin in Western India and Acharya P. C. Ray in Bengal helped to establish the early manufacturing units for galenicals in the latter half of the last century. The import restrictions during the two World Wars gave a fillip to the local industries. By 1930, biologicals, sera, vaccines, anaesthetics, alkaloids, anti-leprosy drugs, etc., were produced ; shark liver oil, glandular products and some sulpha drugs were soon added to the manufacturing list. It is noteworthy that in the face of growing foreign competition, shortage of raw materials and increasing demand for drugs, nearly 70 per cent of the requirements were met by indigenous production in 1943 as compared to the 13 per cent thirty years earlier. The production units and factories existing about that period may be classified as : (1) Government factories ; (2) large scale private enterprises under foreign control and collaboration ; (3) large private enterprises under Indian management ; and (4) small private enterprises. Soon after the War in 1945 local demand for drugs steadily increased, foreign-made specialties flooded Indian market and the more experienced and long-established foreign manufacturers with better technical know-how were having increasing number of distributors and processing units in India. Dependence on foreign imports for basic drugs grew rapidly and the newer developments in chemotherapy were too fast for the none-too-well established Indian pharmaceutical industry. The locally produced items became obsolete and surplus to a large extent. The result was that most of the smaller firms found their position uneconomical, and until a decade ago the main activities of most Indian pharmaceutical manufacturers were confined to ampouling, capsuling, compounding, tabletting and repacking of imported basic and proprietary drugs. Some of the larger firms imported the penultimate products and the last step in the manufacture was done here.

The Antibiotics Industry

The important place accorded to antibiotics and chemotherapeutic drugs in the health and welfare scheme of the nation right from the beginning is reflected in the fact that they accounted for over 75 per cent of the drug imports during 1949-53. The Health Survey and Development Committee, popularly known as the Bhore

INDIA—Value of Drug Imports (in millions of Rs.)

Year	Value of all drugs	Value of antibiotics
1929-30 ..	20.0	—
1949-50 ..	78.5	About 80% antibiotics, sulphas, chemotherapeutics, vitamins
1950-51 ..	105.5	
1951-52 ..	156.0	
1952-53 ..	113.4	
1953-54 ..	123.0	45
1954-55 ..	131.4	41
1955-56 ..	150.0	42
1956-57 ..	149.0	42

Committee, was appointed by the Government of India in 1942 to survey existing health conditions, medical aid and allied matters, and to formulate plans for better medical relief in the country. The Committee recommended among other things, self sufficiency in the production of essential drugs with penicillin, sulpha drugs and antimalarials receiving top priority in the programme. The Government's Panel on Fine Chemicals, Drugs and Pharmaceuticals appointed towards the end of the War drew up a plan to start production of these drugs. About 1948, Government decided to set up a penicillin factory in the public sector after having studied production methods in and having negotiated with, foreign firms. Pending implementation of the project, the Indian Penicillin Committee appointed by the Government of India in 1949, set up a semi-automatic bottling plant in Bombay, the forerunner of Hindustan Antibiotics, at a capital cost of Rs. 4.45 million, to bottle and sell imported bulk penicillin. At this time the UNICEF and WHO offered financial and technical assistance for establishing a penicillin plant in the public sector. The story of the beginnings and achievements of Hindustan Antibiotics is told in detail elsewhere (this *Bulletin*, vol. 1, p. 11, 1958). The plant was designed in 1951 to produce about 3.6 m.m.u. of penicillin with a target of 9 m.m.u. per year. The first normal year of production was 1956-57 although small quantities of finished penicillin were available from 1954-55. By 1956-57 it was also possible to exceed the earlier targets, research and improvements in penicillin technology being the main contributive factors for the success.

Year (Apl.—Mar.)	Finished Penicillin in m.m.u.
1955-56 ..	.. 0.62
1956-57 ..	.. 9.89
1957-58 ..	.. 21.58
1958-59 ..	.. 29.03

In 1953, the Ministry of Commerce and Industry appointed the Pharmaceutical Enquiry Committee to survey the drug industry in India in all aspects and to make suggestions for indigenous production of essential and basic drugs starting from raw materials. In its report (1954) the Committee recommended a number of steps for encouraging local manufacture in the public and private sectors, terms for foreign collaboration, programme for quality control and standardization, distribution and sale, etc., and also the constitution of an advisory Development Council for pharmaceuticals and drugs. Such a council was set up in 1955.

Antibiotics Industry in the Five-Year Plans

The first Five-year Plan did not lay down any specific targets for the production of all pharmaceuticals. However, the target for penicillin for 1955-56 was 4.8 m.m.u. Based on imports and demands in 1952 the Pharmaceutical Enquiry Committee recommended the following capacities for antibiotics :

Antibiotic	Home consumption	Demand	Capacity, end of Plan
Penicillin (m.m.u.)	12.6	20	12.5
Streptomycin (kg.)	9,061	10,000	
Chloramphenicol (kg.)	1,800	2,500	3,600
Chlortetracycline (kg.)	365	400	9,600
Oxytetracycline (kg.)	354	400	
Tetracycline (kg.)			2,400

The present capacity of Hindustan Antibiotics plant is 25 m.m.u., and the present production of penicillin meets about 50 per cent of the country's current demand for the drug estimated at 60 m.m.u. a year. With the extention of medical facilities and concurrent increase in drug requirements, the Development Council recommended the following production capacities :

Antibiotic	Estimated demand before 1958	Recommended capacity 1960-61
Penicillin ..	.. 40-50 m.m.u	45 m.m.u.
Streptomycins	.. 20,000-25,000 kg.	45 tons
Tetracyclines	.. 5,000 kg.	20 tons
Chloramphenicol	.. 7,000-10,000 kg.	10 tons

INDIA—Import of Antibiotics (Value in millions of Rs.)

	1952	1953	1954	1955	1956	1957	1958 Jan.-Nov.
Penicillin	16.5	18.6	16.7	19.7	16.6	17.7	8.96
Streptomycin	14.5	11.5	11.5	14.1	10.8	16.4	9.11
Chloramphenicol	11.0	70	36	37	21	3.99	3.80
Chlortetracycline					11.7	3.46	3.02
Other Antibiotics							4.30

A programme for 60 per cent expansion of the production capacity at Hindustan Antibiotics is already under way at a cost of Rs. 6 million. These additional facilities would be available about July 1959 and output is expected to reach 40 m.m.u. per year thereafter.

In 1956 a seven-man Russian team surveyed the pharmaceutical industry in India. In its report to the Government the team indicated scope for production of all essential drugs including antibiotics from indigenous raw materials. The team estimated the demand for antibiotics to go up steeply by the end of the second plan period following the expansion of health and medical facilities. Their estimates were : penicillin 100 m.m.u., streptomycin 90,000 kg., tetracyclines 40,000 kg., and chloramphenicol 18,000 kg. On this basis the Soviet experts recommended, among other things :

I. Expansion of Hindustan Antibiotics at a cost of Rs. 35 million (*a*) to produce 40 m.m.u. of penicillin per year, (*b*) to build units within the factory for an annual output of 45 tons of streptomycin, 40 tons of tetracyclines and 3 kg. of vitamin B_{12} from antibiotic wastes, and (*c*) production of vitamin D_2 with a capacity of 1,000 kg. per year.

II. Establishment of a new antibiotics plant at a cost of Rs. 85 million for the manufacture of 60 m.m.u. of penicillin, about 45 tons streptomycin, 20 tons new antibiotics, 2 tons vitamin B_2, and 500,000 litres of dextran.

By end of the second Five-year Plan a demand of 75 m.m.u. of penicillin may be expected. In that context, Hindustan Antibiotics could, with certain additions and modifications, increase its production capacity to about 50-60 m.m.u. and, with the installed capacity in the private sector, it should be possible to meet fully the home demand for penicillin.

The requirements of the country for streptomycin and dihydrostreptomycin were at an earlier stage estimated at 40-45 thousand kg. per year, but the demand is growing rapidly and is expected to be about 80 thousand kg. per year. With a view to setting up a plant for the production of these antibiotics at Hindustan Antibiotics a consultant service agreement has been entered into with Merck and Company of U.S.A. Production is expected to start in 1961. The new plant is estimated to cost Rs. 17 million, and the production of 40,000 to 45,000 kg. of streptomycin and dihydrostreptomycin is expected to effect an annual saving of about Rs. 10 million in foreign exchange.

Possibilities for the production of broad spectrum antibiotics—the tetracyclines—are also under investigation and the large scale manufacture of these at Pimpri is also being considered under the second plan.

Licensed schemes in the private sector are : Alembic Chemicals, Baroda, are

establishing a plant for the production of 4.8 m.m.u. of penicillin per annum. Production is expected to start this year.

Standard Pharmaceutical Works, Calcutta, have set up a pilot plant for 1.8 m.m.u. of penicillin per annum. They are also to produce 180 kg. of streptomycin a year.

Parke, Davis and Co., Bombay have been producing chloramphenicol from the penultimate levo base. Their plant capacity of 3,600 kg. per annum is being expanded to 10,000 kg. and steps taken to produce the antibiotic from the next lower intermediate, the nitro base.

Ranbaxy and Co., Faridabad, have been granted licence to set up a plant for 3,000 kg. of chloramphenicol.

Mac Laboratories, Bombay, also have licence for a phased programme for production of chloramphenicol. They will ultimately produce this antibiotic from cinnamyl alcohol imported from Italy. The capacity of their plant is 500 kg. per annum.

Atul Products, Bulsar, have been making chlortetracycline from imported crude chlortetracycline. The company has also started conversion of chlortetracycline to tetracycline. Their installed capacity and production under licences granted are*:

A Calcutta medical firm is reported to have obtained licence for the manufacture of one million vials of antibiotics like penicillin, streptomycin and tetracycline per month. The scheme is expected to cost the company Rs. 1.5 million. The plant and equipment are being purchased from U.K. and U.S.A. on long term deferred payment basis.

In August-October, 1958 an eight-man team of Soviet drug experts toured India to advise the Union Government on the manufacture of medical equipment and drugs. The Soviet Government announced financial aid of 80 million roubles (Rs. 85 million) and technical help and equipment for the manufacture of surgical instruments, drugs, synthetic chemicals, etc. India Government's contribution for these projects may be very much higher. Items for production will be taken up on the basis of priorities established after taking into account accomplishments in the field so far.

Conclusion

When the projects on hand and those envisaged materialize there will accrue a number of direct and indirect benefits to the country. The value of pharmaceuticals produced in India has more than doubled in five years (Rs. 240 million in 1953, 450 million in 1956-57, 620 million in 1957-58). By the end of the second plan period there would be a saving of Rs. 50 to 60 million in foreign exchange, and the prices of

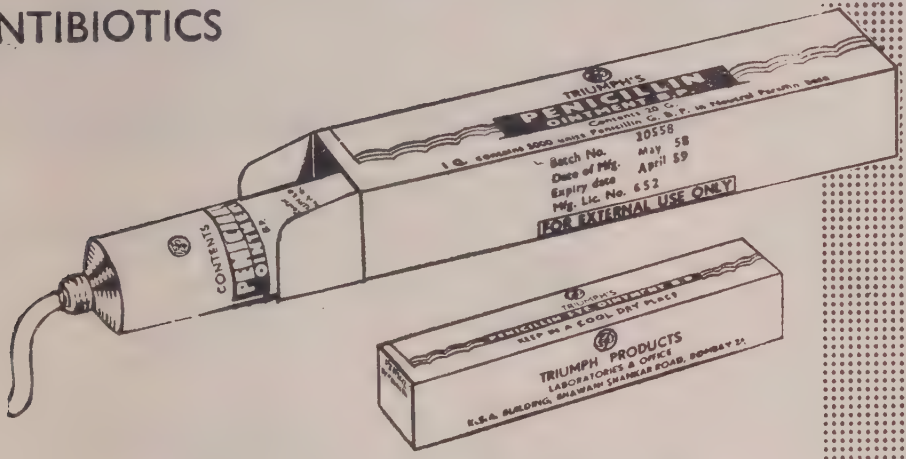
Antibiotic	Installed capacity (kg.)	Production (kg.)					
		1953	1954	1955	1956	1957	1958
Aureomycin HCl	5,200	528.6	696.68	422.1	710.89	1,383.5	1,770.3
Achromycin HCl	3,000	—	—	209.16	295.24	497.7	684.63
Tetracycline primary, neutral	3,000	—	—	—	—	81.17	278.05

* Courtesy : Atul Products.

essential drugs like antibiotics would come down. Export of surplus drugs is not ruled out ; as a matter of fact in 1957 India's export of certain antibiotics was valued at Rs. 5.6 lakhs, the drug consignments being directed mainly to East Africa, Egypt, West Asia and South East Asian countries. Indirectly, there will be a fillip to other industries and stimulation to start new ones to produce the raw materials, intermediates and equipment needed by the pharmaceutical industry. In 1957 the National Industrial Development Corporation listed tentative capacities for production of a number of such items. Already on stream are a bottle and glass factory, an aluminium foil plant, a cardboard plant and a firm for rubber stoppers. The general progress of industrialization will provide greater employment opportunities and help to raise the standard of living in the country.

A.N.

A FURTHER STRIDE IN
MANUFACTURE OF PHARMACEUTICALS
& ANTIBIOTICS



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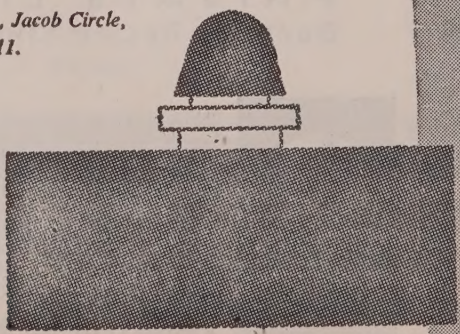

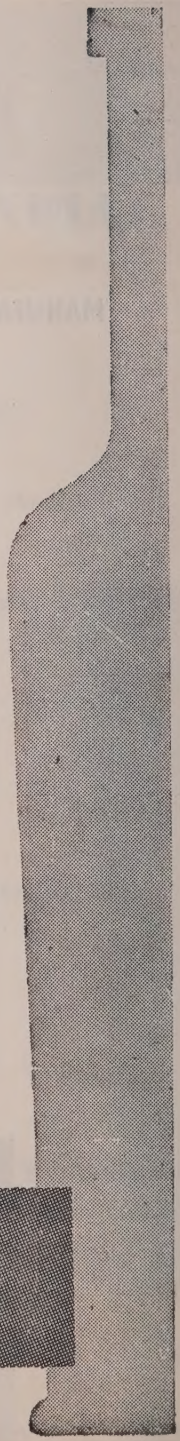

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